Title

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Journal

Behavioral Ecology and Sociobiology, 46(5)

ISSN

0340-5443

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Publication Date

1999

DOI

10.1007/s002650050630

Behav Ecol Sociobiol (1999) 46: 357–365

ORIGINAL ARTICLE

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The genetic mating system of a sex-role-reversed pipefish (*Syngnathus typhle*): a molecular inquiry

Received: 19 January 1999 / Received in revised form: 15 April 1999 / Accepted: 9 May 1999

Abstract In the pipefish *Syngnathus typhle* as in other species of Syngnathidae, developing embryos are reared on the male's ventral surface. Although much laboratory research has been directed toward understanding sexual selection in this sex-role-reversed species, few studies have addressed the mating behavior of *S. typhle* in the wild, and none has capitalized upon the power of molecular genetic assays. Here we present the first direct assessment of the genetic mating system of *S. typhle* in nature. Novel microsatellite loci were cloned and characterized from this species, and employed to assay entire broods from 30 pregnant, field-captured males. Genetic analysis of 1340 embryos revealed that 1–6 females (mean = 3.1) contributed to each brooded clutch, the highest rate of multiple maternity yet documented in any pipefish. Evidence of multiple mating by females was also detected. Thus, this population of *S. typhle* displays a polygynandrous mating system, a finding consistent with previous field and laboratory observations. Our results, considered together with similar studies of other syngnathid species, provide preliminary support for the hypothesis that the genetic mating system is related to the evolution of sexual dimorphism in the fish family Syngnathidae.

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Key words Mating system · Polygynandry · Microsatellites · Parentage · Pipefish · *Syngnathus typhle*

Introduction

In pipefishes and seahorses (family Syngnathidae), males provide all parental care. During copulation, a female transfers eggs to a male's ventral surface where they are fertilized (Vincent et al. 1992). In some species such as seahorses, eggs are deposited through a small opening into an enclosed pouch, whereas in others, the eggs are glued externally on the male's body (Herald 1959). In the pipefish *Syngnathus typhle*, males possess a pouch consisting of two ventral folds in which fertilization occurs, thereby ensuring paternity for the brooding male (Berglund et al. 1986a, 1986b; Jones and Avise 1997b). Females have the potential for a higher reproductive rate than males, and this species is sex role reversed (i.e., competition for mates is more intense among females than among males; Berglund et al. 1986a, 1986b, 1989; Vincent et al. 1992). Sex role reversal provides unique opportunities to test sexual selection theory (Williams 1975; Andersson 1994), and *S. typhle* has been exploited in numerous laboratory studies to investigate various aspects of sexual selection and mating behavior (e.g., Ahnesjö 1992a, 1992b; Berglund 1995; Rosenqvist and Johansson 1995; Fuller and Berglund 1996).

Despite detailed laboratory analyses as well as several field studies focusing on *S. typhle* reproductive behavior (Vincent et al. 1994, 1995), the natural mating system of this species has remained largely unexplored. Laboratory observations have shown that *S. typhle* females and males seek multiple mates during the course of a single male pregnancy (Berglund et al. 1988, 1989; Berglund and Rosenqvist 1990), and that females produce more eggs than the male broodpouches can accommodate (Berglund et al. 1989; Berglund and Rosenqvist 1990). This reproductive inequality results in a female-biased

operational sex ratio, causing the above mentioned sex role reversal (Clutton-Brock and Vincent 1991; Clutton-Brock and Parker 1992). In support of laboratory observations, pregnant males from field samples often contain differently colored eggs (Berglund et al. 1988; Vincent et al. 1995) that presumably originated from different females. However, extrapolations based on either laboratory observations or egg color have the potential to misrepresent the natural genetic mating system (Jones and Avise 1997b).

Empirical data on genetic mating systems in nature were lacking until molecular markers permitted detailed analyses of parentage in various groups such as birds (Birkhead and Møller 1992; Avise 1994; Westneat and Webster 1994). Although the relevance of mating pattern to the operation of sexual selection had long been appreciated (Darwin 1871; Clutton-Brock et al. 1980; Payne 1984; Björklund 1990; Oakes 1992), molecular studies of parentage underscored the need to distinguish between the social mating system and the genetic mating system (Møller and Birkhead 1994; Avise 1996). Indeed, the latter is often more germane to the operation of sexual selection (Andersson 1994). For example, in genetically polygynous species with conventional sex roles, intense sexual selection can lead to the evolution of secondary sexual characters in males (Darwin 1871; Andersson 1994). The analogous prediction in genetically polyandrous species with reversed sex roles is that intense sexual selection may promote the evolution of secondary sexual characters in females (Jehl and Murray 1986; Andersson 1995). Despite the opportunities that sex-role-reversed taxa afford in interpreting sexual selection theory (Williams 1975), few studies have used molecular methods to investigate mating systems in such organisms (Oring et al. 1992; Jones and Avise 1997a; Delehanty et al. 1998).

Microsatellite markers are well suited to questions of parentage in fishes (Kellogg et al. 1995, 1998; Colbourne et al. 1996; Parker and Kornfield 1996; Jones and Avise 1997a, 1997b; Jones et al. 1998a, 1998b). Knowledge of the genetic mating system of *S. typhle* will permit interpretations of past and future laboratory studies of this species in the context of natural mating behavior. The genetic mating system of *S. typhle* is also of interest in a relational context. Compared to other syngnathids, *S. typhle* exhibits only modest apparent sexual dimorphism. Males and females are phenotypically similar except during courtship and mating when females assume transient ornamentation patterns (Berglund et al. 1997; Bernet et al. 1998). Comparisons between the genetic mating system and the degree of sexual dimorphism across syngnathid species will contribute to a continuing exploration of the interplay between mating system evolution and the operation of sexual selection in this group of male-pregnant fishes (Jones and Avise 1997a, 1997b; Jones et al. 1998a).

The current goals have been to develop and employ polymorphic microsatellite markers to investigate the genetic mating system of wild-caught *S. typhle*. The approach has permitted the following: (1) determination of the rate of multiple mating by males; (2) documentation of multiple mating by females; (3) evaluation of a hypothesis, based on laboratory observations and the extent of sexual dimorphism, that *S. typhle* should be polygynandrous, and (4) a comparison of the results to the genetic mating systems of other previously studied syngnathids.

Methods

Collection of field samples

Adult males were collected 6 and 8 July 1996, from Gullmar Fjord on the Swedish west coast (58°15′N, 11°28′E) by pulling a small beam trawl (2-mm mesh) behind a boat through shallow (0.5–6.0 m) eelgrass meadows. Forty-four pregnant males were caught and returned live to Klubban Biological Station where they were frozen and held at −70 °C until they were transported on dry ice to the University of Georgia for molecular analysis.

Tissue samples were prepared for the polymerase chain reaction (PCR) as in Jones and Avise (1997a). To assay individual embryos, we dissected away the outer membrane of the father's brood pouch and removed each embryo with forceps, mapping its position within the brood pouch as it was extracted. For embryos with large, easily visible yolks, we recorded yolk color before removing the yolks with forceps. Embryos were rinsed in deionized water and placed individually in microcentrifuge tubes to which 50–150 µl of Gloor and Engels' (1992) fly buffer was added. Samples were incubated for 30 min at 37 °C followed by 2 min at 95 °C. After a 2-min centrifugation at high speed in a microcentrifuge, 2 µl of the resulting supernatant was used for PCR. For adults, the same procedure employed either a caudal fin clip or a small tissue sample from the brood pouch.

Microsatellite characterization

We first attempted to amplify microsatellites from *S. typhle* using PCR primers developed previously from the gulf pipefish *S. scovelli* (Jones and Avise 1997a), because these primers had also revealed polymorphic loci in the dusky pipefish *S. floridae* (Jones and Avise 1997b). These primer-pair sequences (not previously published) are presented in Table 1. One primer pair (*micro25.22*) failed to yield a consistent amplification product in *S. typhle*. The other three primer pairs produced fragments of the expected size, but levels of polymorphism were too low (1–3 alleles per locus; Table 1) for further utilization in the current study. Thus, novel microsatellite primers for *S. typhle* were developed.

Genomic DNA was extracted from a single *S. typhle* individual using a standard proteinase K, phenol:chloroform protocol. The DNA was digested with *Mbo*I and 200- to 700-bp fragments were isolated by excision from a 2% agarose gel. This purified insert DNA was then ligated into *Bam*HI-digested, dephosphorylated pBluescript phagemid (Stratagene), and the resulting ligation was transformed into heat-shock-competent XL1-Blue *Escherichia coli* (Stratagene). This partial genomic library was screened with two cocktails of synthetic oligonucleotides containing: (a) $(GT)_{10}$, $(GGAT)_4$, $(GACA)_4$, and $(TAG)_6$; (b) $(GATA)_4$, $(GA)_{10}$, $(TCC)_5$, and $(TTAGGG)_3$. Inserts of positive clones were sequenced using the fmol DNA Sequencing System (Promega), and PCR primers were designed to amplify six of the identified microsatellite loci (Table 1).

Microsatellites were amplified in 10-µl reaction volumes containing 1 × Promega Taq buffer, 1.25–1.50 mM $MgCl_2$, 0.15 µM

Table 1 Microsatellite loci assayed from random samples of adult *Syngnathus typhle*. Four of the loci were originally cloned from and were highly polymorphic in the gulf pipefish *S. scovelli* (Jones and Avise 1997a). The remaining six loci were cloned from *S. typhle*. Shown are the original cloned repeat, primer sequences, sample size (*n*), number of alleles, and the observed and expected heterozygosities (*na* primers for this locus failed to yield consistent amplification products in *S. typhle*)

Locus [cloned repeat]	Primer sequences (5′→3′)	*n*	Number of alleles	Heterozygosity	
				Observed	Expected
Syngnathus scovelli microsatellite loci					
micro25.10	ATTGCGTTCAGTGTTGTCATTTTCTA	11	2	0.273	0.247
$[(CA)_4CG(CA)_6]$[a]	GAGTGACTCGGGCTTCGGTTAC				
micro25.22	ACGTCACTCACGCCTCTGCC	11	na	na	na
$[(GT)_{10}]$	CGACTCACCCTTATAACTTCTCAATC				
micro11.1	GCATTAGTCTCTATGTTGACCTTTCC	11	3	0.546	0.558
$[(GT)_{12}GC(GT)_{12}]$[a]	CAATCCAAGATCACATGATTTGTTAC				
micro22.3	CTGCTGCATGTTTGTGTATGAAA	11	1	0	0
$[(CA)_{39}]$	ATTGCTTCTCCCTCTGAAGTCTC				
Syngnathus typhle microsatellite loci					
*typh*04	CTGGAAGCAGACTTTTATGGG	293	39	0.949	0.950
$[(GGTT)_5(GGAT)_6]$	GCTGTTTTTGCTACCACACCA				
*typh*12	GCGTCCCATTCACTGACTTGATTG	66	15	0.546	0.599
$[(CA)_{12}CT(CA)_5]$	CCCCATGCTTCAGGCTTTCACTAT				
*typh*16	CAGGACACGCTGGAAAGAC	293	20	0.925	0.918
$[(GATG)_{15}]$	GCAACACCTTGAAGAGGAAAGT				
*typh*18	GCAGGGATGAAGTGACAATG	64	38	0.953	0.967
$[(GA)_{51}]$[a]	TTGAGATAAATAAATGGTGTTCTAAAG				
typh33	TACATGGGTGAGTCTTAGGGT	8	1	0	0
$[(GT)_7]$	TTACGCACAAGGCTATTTCAT				
typh44	TGCCATCTTGCTGATAAGTTG	8	1	0	0
$[(GT)_5CT(GT)_6]$	AAAACTGATGAGGAACTCTGA				

[a] These microsatellite loci were flanked by 6–34 bp of additional sequence containing imperfect microsatellite repeats

of each primer, 0.1 mM of each dNTP, and 0.5 units of Promega Taq polymerase I. One primer was end-labeled with 1 μCi [γ^{32}P]-ATP per 5 pmol prior to PCR. Cycling parameters consisted of an initial denaturation at 94 °C for 2 min, followed by 30 cycles at 94 °C for 1 min, at an optimal annealing temperature for 1 min, and at 72 °C for 1 min. A final extension at 72 °C for 4 min concluded the thermal cycling. Loci *typh*04 and *typh*16 were multiplexed by adding primers for both loci to a single PCR with 1.5 mM $MgCl_2$, and by using an optimal annealing temperature of 61 °C. Loci *typh*12 and *typh*18 were amplified separately at 55 °C with 1.50 mM and 1.25 mM $MgCl_2$, respectively. PCR products were resolved on standard denaturing 6% polyacrylamide gels and visualized by overnight autoradiography. Field-collected adults of both sexes were assayed at the four loci to establish population allele frequencies (Table 1; Fig. 1).

Maternity analysis

Genotypes at *typh*04 and *typh*16 were determined successfully for 30 pregnant males and for 1340 embryos (among 1344 total) contained within their brood pouches. For the four embryos for which the PCR failed, we assumed that the untyped embryo had the same mother as neighboring embryos. Microsatellite loci *typh*12 and *typh*18 were used to resolve ambiguities and to establish four-locus genotypes for inferred mothers of special interest.

Multi-locus maternal genotypes were reconstructed by examining the associations of alleles across loci (as described in Jones and Avise 1997b). These maternal genotypes are to be interpreted as the minimum number of females necessary to explain the data; the presence of an additional female's gametes was invoked only in cases that could not be explained by a null allele or a de novo mutation (see below). For microsatellite loci with levels of variability similar to those used here, the minimum number of mothers should be very close to the actual number (see discussions in Jones and Avise 1997a, 1997b; Jones et al. 1998a, 1998b).

Results

The microsatellite markers

Four microsatellite loci cloned from *S. typhle* proved to be polymorphic (Table 1; Fig. 1), displaying from 15 to 39 alleles each and exhibiting heterozygosities ranging from 0.55 to 0.97. The two apparently monomorphic *S. typhle* loci (*typh*33 and *typh*44) contained relatively short microsatellite arrays (Table 1). All loci were in Hardy-Weinberg equilibrium in the adult population sample (exact test in GENEPOP; Raymond and Rousset 1995), without significant genotypic disequilibria detected among pairs of loci (GENEPOP exact test). Two of the newly developed *S. typhle* microsatellite loci (*typh*04 and *typh*16) involved tetranucleotide repeats, had low background sub-banding on gels, and displayed non-overlapping sizes such that they could be multiplexed and scored relatively rapidly.

Non-amplifying (null) alleles can potentially complicate microsatellite-based assessments of parentage. In studies such as this involving large numbers of offspring per adult, null alleles are apparent from the "non-Mendelian" transmission of alleles from parents to progeny (Jones and Avise 1997a). From such appraisals, the presence of a null allele was inferred at *typh*04, but it was far too rare in the population (frequency = 0.003) to complicate mating system analysis. Null alleles were not detected at the other loci, and invoking their occasional

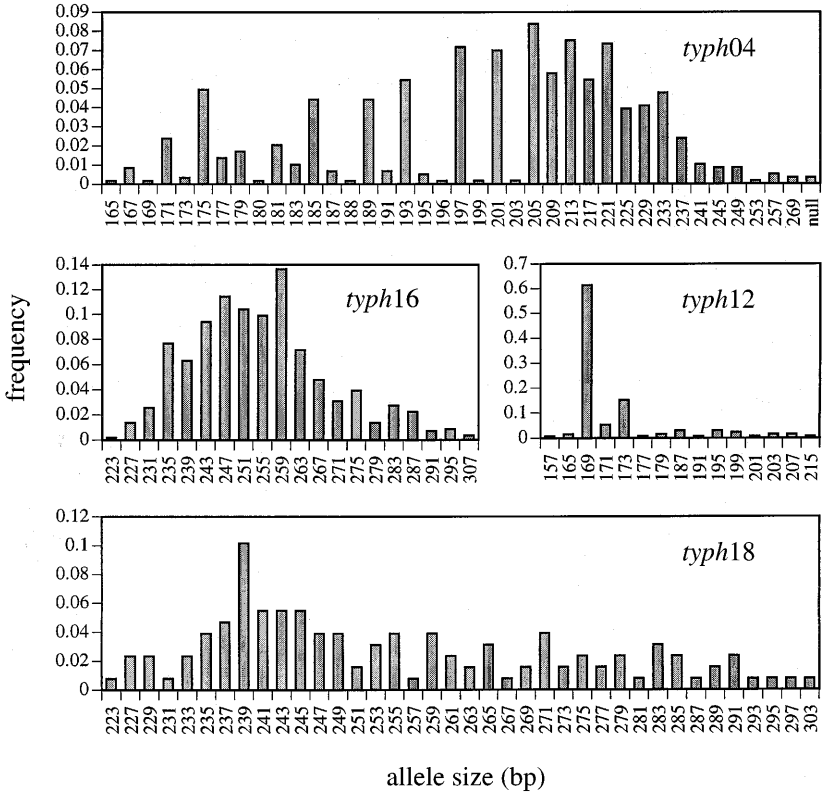

Fig. 1 Allele frequency histograms for the four novel *Syngnathus typhle* microsatellites employed in this study. Alleles are designated by size (in bp) of the PCR product produced by the primers. Frequencies are based on sample sizes given in Table 1

presence would not change the results because most individuals were heterozygous for detectable alleles.

Mutations are another potential source of error in parentage studies. De novo mutations were not observed at *typh*12 or *typh*18, but apparent germ-line mutations were detected for *typh*04 and *typh*16. Mutations in the paternal germ-line were indicated by progeny that failed to receive an allele identical in state to either of the brooding father's alleles. In principle, such events might be interpreted alternatively as paternity exclusions of the brooding father. However, this possibility is extremely unlikely in the present cases because (a) the "exclusions" occurred as only one or a few embryos in a male's brood, (b) they were never supported by evidence from additional loci, and (c) egg fertilization in this species normally occurs internally, within the male's brood pouch.

Detection of mutations arising in the female germline is more problematic because maternal genotypes in a progeny array are deduced by subtraction of the known paternal genotype. Our conservative rule of thumb was to invoke a maternal-origin mutation only when the following criteria were met: (1) a single embryo in the brood pouch displayed a genotype inconsistent with its being the progeny of any of the inferred mothers of the other embryos; (2) the inconsistent genotype was due to a single allele at one locus, and (3) data from the other three loci were consistent with the embryo in question being the progeny of one of the inferred mothers of the other progeny.

The results of a broader survey of mutations at these loci will be presented elsewhere (Jones et al., in press). In the present study, 13 apparent mutations were observed for *typh*04 and 2 were observed for *typh*16, yielding mutation rate estimates of 4.9×10^{-3} and 7.5×10^{-4}, respectively. Four of these mutations were of maternal origin, whereas 11 occurred in the paternal germ line. Assuming that mutant alleles arose from the nearest-sized alleles in the parent, 12 mutations involved insertions or deletions of 4 bp, and 3 involved insertions or deletions of 8 bp. All except one of the observed mutations resulted in alleles that were identical in size to a preexisting allele in the population. Thus, only rarely were novel size variants generated by de novo mutations. Despite the modest number of mutations detected, the mating system analyses were not compromised because most mutations occurred at one locus (*typh*04), a large number of progeny per family was assayed, and the assessments of paternity and maternity were based on information from multiple loci.

Male mating behavior

All field-collected adult males were pregnant. Except for occasional abnormal progeny (see below), embryos within a brood pouch were of approximately the same developmental stage, and we assayed only males whose pouches contained embryos with visible eyes. On average, males contained 44.8 embryos (range 23–65; Table 2). Males ranged in length from 111 to 165 mm (mean 136.4 mm; Table 2). We found no significant

Table 2 Summary of the 30 *S. typhle* males assayed. Shown are a male's ID, his standard length, and the total number of embryos (with the subset of these that were abnormal shown in parentheses) from his brood pouch. We attempted PCR on all 1344 embryos, including the abnormal ones. The fourth column shows the number of additional undeveloped embryos from each male's pouch. These were not amplified. Also shown is the minimum number of mates per male inferred from the microsatellite genotypes of brooded progeny. The final column shows the number of embryos deposited by each inferred mother, in order from the presumed first to mate (embryos most posterior in the pouch) to last to mate (anterior embryos)

Male ID	Length (mm)	Number of embryos (abnormal)	Undeveloped embryos	Number of mates	Number of embryos from each female (first to last)
FCST2	135	23 (0)	0	2	16, 7
FCST3	141	43 (1)	6	1	43
FCST4	111	37 (0)	0	5	9, 14, 3, 4, 7
FCST6	131	60 (1)	3	3	46, 2, 12
FCST7	165	63 (0)	1	4	9, 31, 1, 22
FCST8	131	44 (7)	9	2	14, 30
FCST9	160	62 (0)	0	3	6, 26, 30
FCST10	137	30 (0)	6	3	8, 13, 9
FCST11	127	42 (0)	2	2	26, 16
FCST12	131	62 (0)	0	3	16, 36, 10
FCST13	130	27 (0)	0	4	4, 3, 19, 1
FCST17	135	54 (0)	5	4	20, 30, 2, 2
FCST18	148	50 (1)	2	3	17, 8, 25
FCST19	133	24 (0)	3	3	14, 2, 8
FCST20	122	42 (0)	0	1	42
FCST22	142	39 (0)	1	3	1, 23, 15
FCST24	131	56 (1)	4	3	13, 36, 7
FCST25	131	36 (1)	7	3	4, 29, 3
FCST26	132	59 (0)	2	1	59
FCST27	141	42 (0)	1	3	17, 15, 10
FCST29	124	57 (2)	3	6	4, 5, 11, 14, 16, 7
FCST30	124	45 (0)	2	4	19, 2, 18, 6
FCST34	126	27 (1)	0	2	22, 5
FCST35	163	65 (1)	0	5	2, 11, 11, 25, 16
FCST36	162	23 (0)	1	4	10, 4, 5, 4
FCST39	124	44 (0)	0	3	10, 10, 24
FCST40	138	45 (1)	0	4	2, 5, 30, 8
FCST42	142	52 (0)	1	2	24, 28
FCST43	127	41 (0)	0	3	4, 15, 22
FCST55	149	50 (0)	0	4	14, 1, 30, 5

relationship between male length and brood size ($r = 0.27$, $n = 30$, $P = 0.15$).

In addition to normally developing young, the pouches of many males contained what appeared to be either undeveloped embryos or unfertilized eggs (Table 2). We tested these for spatial non-randomness in intra-pouch distributions by dividing a male's pouch into thirds and comparing observed to expected numbers. By this measure, no detectable systematic bias in the distribution of undeveloped eggs was detected ($\chi^2 = 2.95$, $df = 2$, $P > 0.10$). Some males also contained developmentally abnormal embryos (Table 2) that were usually smaller than the others and frequently lacked eyes. However, these embryos exhibited normal genotypes (i.e., those expected given the parents) at our microsatellite loci.

Notwithstanding the single-locus mutations noted above, all embryos displayed genotypes consistent with their having been sired by the male in whose pouch they were present. Thus, as in other syngnathid species studied genetically (Jones and Avise 1997a, 1997b; Jones et al. 1998a), *S. typhle* males have a complete confidence of paternity in their brooded embryos. This paternity assurance facilitates determination (by subtraction) of the maternal alleles contributed to an embryo. Evidence of multiple maternity was found within 90% of the broods assayed. The mean number of successful mates per male was 3.1 (range 1–6; Table 2; Fig. 2). Within each brood with multiple mothers, embryos were clumped spatially by maternity within a brood pouch (Fig. 3). Surprisingly, in nine (30%) of the males, two or three mothers contributed a substantial number of eggs to the brood whereas an additional female mothered only one or two embryos (Fig. 3, Table 2). These results were supported by two or more loci. We found no significant correlation between the length of a male and his number of mates ($r = 0.14$, $n = 30$, $P = 0.46$) or between a male's number of mates and the number of embryos in his brood pouch ($r = 0.16$, $n = 30$, $P = 0.39$).

Female mating behavior

As in other microsatellite-based parentage studies of pipefish, probable multiple mating by females was detected. The evidence consists of the appearance of identical multi-locus maternal genotypes deduced for progeny in the pouches of different males. In principle,

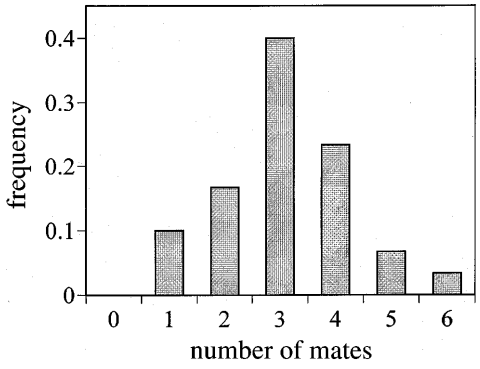

Fig. 2 Frequency histogram showing the numbers of genetically inferred mothers per brood in the progeny of pregnant *S. typhle* males

an alternative explanation for such genetic matches invokes the possibility that two separate females with identical genotype deposited eggs in the pouches of different males. However, expected frequencies of the four-locus inferred genotypes of the females involved were sufficiently low (2.6×10^{-11}–3.3×10^{-7}; Fig. 4) that this hypothesis can be rejected. Thus, on four occasions in our sample, an individual female almost certainly deposited eggs in the pouches of two different males (Fig. 4).

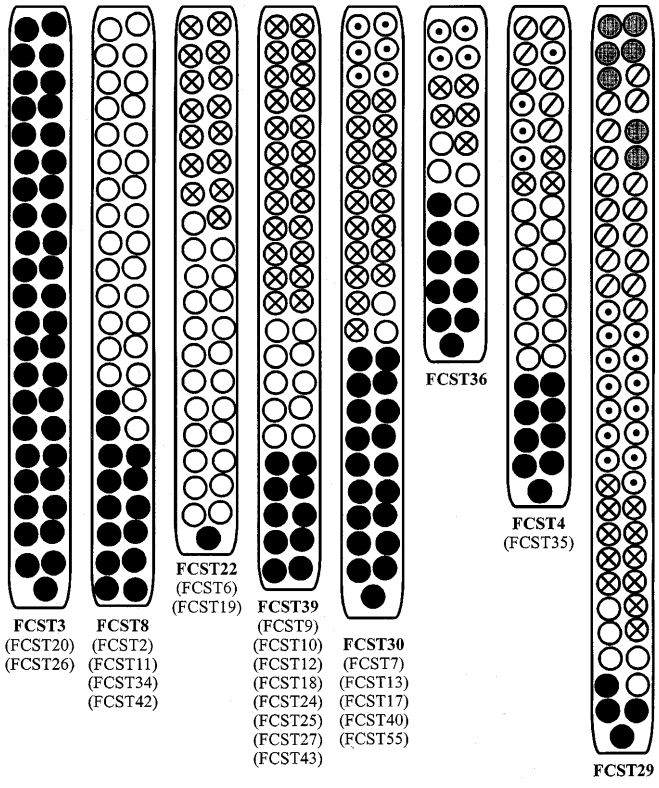

Fig. 3 Diagrammatic representation of eight typical patterns of parentage observed in the brood pouches of *S. typhle* males. Each *rectangle* represents a brood pouch on the male's ventral surface (anterior toward top). Each *circle* is an embryo. Those embryos with the same pattern within a brood were inferred by genotype to be full sibs; those of different patterns were half sibs (had different mothers). Embryos from different broods were generally unrelated (Fig. 4 displays exceptions). Embryos are shown in two rows for simplicity (in reality, several rows could be present). Patterns in the third and fifth pouches from the left depict outcomes in which a substantial number of embryos derive from two or three mothers whereas an additional female contributed only one or two eggs to a brood. Codes in **bold** indicate the identity of the male whose pouch is shown, while codes in *parentheses* indicate additional males whose broods displayed similar patterns of maternity (Table 2)

Discussion

The genetic mating system of *S. typhle*

The current genetic analysis of a natural population of the pipefish *S. typhle* indicates that 90% of the brood pouches of pregnant males contained eggs from multiple females. Assayed males mated with as many as six females, a level of multiple maternity substantially higher than reported in other syngnathid species (Jones and Avise 1997a, 1997b; Jones et al. 1998a). We also provide documentation that female *S. typhle* in nature have multiple mates. Given that only a small portion of the breeding population at this locale was sampled, and that females may have mated with additional males not collected, our results probably severely underestimate the true rate of multiple mating by females. Given this inherent sampling bias, the fact that multiple mating by females was detected at all suggests that females frequently mate with multiple males (Jones and Avise 1997a, 1997b). Thus, the genetic mating system of *S. typhle* is best described as polygynandrous.

Expectations based on previous field and laboratory studies of *S. typhle* mating behavior are supported by the current genetic data. Males generally mate with multiple females under laboratory conditions (Berglund et al. 1986a, 1986b, 1988). Furthermore, field-caught males frequently contain eggs of different colors that presumably originated from different females (Berglund et al. 1988; Vincent et al. 1995). Based on visual inspection of egg color, Vincent et al. (1995) found that males appeared to mate with a mean of 3.5 females (range 1–7), a number slightly exceeding our genetically based estimate of 3.1 mates per male (range 1–6). The visual difference among embryos from different mothers is due to variable egg-yolk colors that fade as progeny develop and the yolk is absorbed. Twenty-one of our males were too advanced in pregnancy for yolk color to be informative. For the remaining 9 males, egg color differences yielded an estimate of 2.3 mates per male, somewhat lower than the estimate of 3.2 mothers per brood based on the genetic analyses for these males (paired *t*-test, $P = 0.01$). This result suggests that if the pregnant males studied by Vincent et al. (1995) had been assayed genetically, an even higher incidence of multiple maternity might have been recorded.

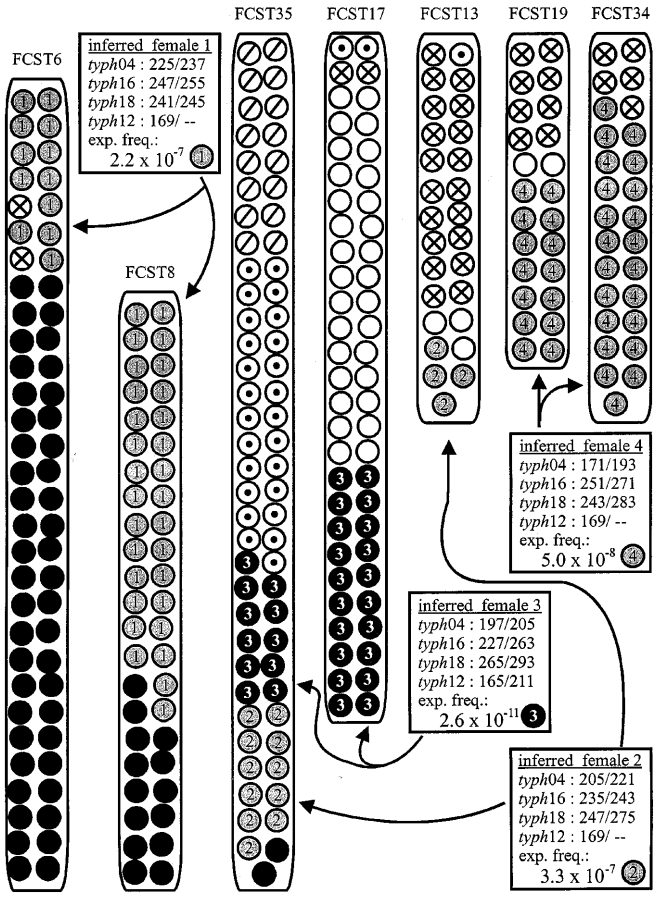

Fig. 4 Graphical representation of the four detected cases of multiple mating by a female. The brood pouches of males and their embryos are as shown in Fig. 3. Non-numbered embryos from different pouches were unrelated. Same-numbered embryos from different pouches shared a maternal genotype. The low expected frequencies of these multi-locus genotypes (see *boxes*) suggest in each case that one female contributed (as shown by *arrows*) to the brood pouches of two males. *Dashes* indicate instances in which only one maternal allele was observed in the assayed progeny. In these cases, the mother could have been either homozygous, or heterozygous for the observed allele and another allele that failed to segregate in the progeny

The higher number of mates per male observed by Vincent et al. (1995) could be explained by several factors. First, more than half of the males studied by Vincent et al. (1995) were longer than 170 mm, whereas all of our males were less than 170 mm (mean 136 mm). Although neither study found a correlation between male size and number of mates (which would explain the discrepancy), the data do not conclusively rule out such a relationship. Second, our sample was taken at the end of the first brooding period during the summer. Most males mate at least twice during the summer, and the operational sex ratio (OSR) changes dramatically during the breeding season (Vincent et al. 1994). Such fluctuations in OSR probably affect mating behavior (Berglund 1994) and may also affect rates of multiple maternity. Finally, Vincent et al. (1995) looked at eggs early in pregnancy, when egg color may be a more reliable guide to rates of multiple mating by males (since egg color fades) and brood reduction (see below) has not yet occurred. Thus, future work might address the relationship between male size and number of mates as well as temporal changes in the mating system as the OSR fluctuates.

A surprising pattern is that pregnant males in our sample frequently contained only one or two embryos that originated from an additional mother. Reasons for this outcome remain uncertain. In *S. typhle*, mating is an involved process (Fiedler 1954). Thus, it seems unlikely that an adult would initiate courtship only to donate or accept a single egg. Perhaps mating events are often interrupted in nature. Another possibility is that after egg transfer is initiated, one or the other partner re-evaluates mate quality and terminates copulation before many eggs are exchanged. A third possibility is that such singletons or pairs of embryos represent the signature of brood reduction. The number of progeny at the end of a male's pregnancy is often substantially smaller than the number of eggs initially received (Ahnesjö 1992a), and the probability of embryo survival during the brooding period may be related to its maternity (Ahnesjö 1996). Thus, the pattern noted above may reflect a reduction during pregnancy from a large number of deposited eggs to a much smaller number of surviving progeny. Undeveloped embryos frequently observed in a male's pouch may be a residual consequence of such brood reduction.

Another intriguing possibility that may explain the occasional presence within the brood pouch of only one or two eggs from a given mother stems from an observation on field courtship (Vincent et al. 1995). During egg transfer between a large female and a receptive male, smaller females may attempt to mate with the male by positioning their ovipositors near the male's pouch opening. Vincent et al. (1995) did not observe egg transfer between smaller females and a receptive male. However, if such a "sneaker" female was occasionally successful, she might be expected to transfer only one or a few eggs, resulting in the genetic pattern we detected. This situation would be the role-reversed analog of sneaking behavior in other fish species in which a satellite or sneaker male may fertilize some of the eggs in a bourgeois male's brood (Taborsky 1994).

Comparisons to other syngnathid species

The genetic mating system of *S. typhle* differs substantially from those deduced for some other syngnathid species using similar molecular markers. Assayed males of the Gulf pipefish *S. scovelli* and the Western Australian seahorse *Hippocampus angustus* generally receive eggs from only one female during the course of a pregnancy (Jones and Avise 1997a; Jones et al. 1998a). The brood pouch of an assayed dusky pipefish (*S. floridae*) male typically contains embryos mothered by one to three females (Jones and Avise 1997b), a level of multiple maternity substantially lower than seen in *S. typhle*. Furthermore, evidence for multiple mating by females has been observed for all *Syngnathus* species studied

genetically to date (Jones and Avise 1997a, 1997b), but not for the apparently monogamous *H. angustus* (Jones et al. 1998a). Given these diverse syngnathid mating systems, the question arises as to why *S. typhle* males mate with so many females.

In species with conventional sex roles, such as most mammals, the benefits to males of multiple mating are obvious – through such behavior, a male may increase his reproductive success with little cost. However, in sex-role-reversed species such as pipefishes, in which males are limited severely by intrinsic reproductive capacity, the benefits of multiple mating are less obvious. Many hypotheses have been proposed to explain multiple mating by females in species with conventional sex roles (Reynolds 1996). Some of the same hypotheses may be applicable to some degree for both sexes of *S. typhle*.

Berglund et al. (1988) discussed four selection hypotheses for multiple mating in *S. typhle*: (1) reduced copulation time may decrease the risk of predation; (2) increased genetic variability in broods may benefit one or both parents; (3) females may reduce their risk of producing no surviving offspring by spreading eggs among multiple males, or (4) females may benefit from the allocation pattern by reducing competition among their offspring within the brood pouch. Berglund et al. (1988) concluded that the pattern of reproduction in *S. typhle* is probably driven by the last two hypotheses. Namely, females are "bet hedging" by spreading eggs among multiple males, and large females benefit by depositing a small number of eggs per male to reduce competition among siblings within the brood pouch. Among other hypotheses yet to be addressed include the possibilities that (1) individuals may better assess mate quality by copulating with additional mates, or (2) males may seek multiple mates to increase egg competition during brood reduction and thereby obtain higher-quality offspring.

Although one or more of these hypotheses may explain polygynandry in *S. typhle*, why have presumably similar selective constraints not yielded similar mating systems in all syngnathids? The answers are probably to be found in ecological factors affecting these species differentially (Emlen and Oring 1977). For example, some authors have suggested that adult population density may affect the mating system, with low density favoring the appearance of monogamy (e.g., in seahorses; Vincent et al. 1992). Another dramatic ecological difference among species is the length of the mating season. In the warm-temperate populations of *S. scovelli* and *S. floridae*, mating probably continues year round (J. Brown, unpublished data), whereas the high-latitude *S. typhle* breeds for only a short period in summer. Perhaps a longer breeding season reduces the selective pressure for a bet-hedging strategy by females. Many other factors may shape the genetic mating system, but too little information is available regarding reproductive ecology in most syngnathids to effectively evaluate competing hypotheses.

The question of mating system evolution is important in understanding the operation of sexual selection and the evolution of sexual dimorphism (Andersson 1994). In the Syngnathidae, all species of which exhibit male pregnancy, there appears to be a relationship between the mating system and sex role reversal (Vincent et al. 1992). Thus, monogamous species tend not to be sex role reversed (by the definitional criterion of a gender-based asymmetry wherein competition for mates is more intense among males than among females). Conversely, polygamous species tend to be sex role reversed (females compete more intensely for mates than do males). Furthermore, the available data on syngnathid genetic mating systems are consistent with the hypothesis that the more polyandrous species exhibit greater levels of sexual dimorphism (with females being the sexually selected sex) than those that are less polyandrous (i.e., are either monogamous or polygynandrous). Thus, *S. scovelli* is extremely dimorphic and has a primarily polyandrous genetic mating system (Jones and Avise 1997a), *H. angustus* appears to be monogamous and is sexually monomorphic (Jones et al. 1998a), and both *S. typhle* and *S. floridae* exhibit moderate levels of sexual dimorphism and are genetically polygynandrous (Jones and Avise 1997b).

Acknowledgements We thank M. Sandvik for help in the field and B. Nelson for assistance in the laboratory. Work was supported by the National Science Foundation (A.G.J., J.C.A.), a National Institute of Health Training Grant (A.G.J.), a Pew Fellowship and University of Georgia funds (J.C.A.), a Norwegian Research Council Grant (G.R.), and a Swedish Natural Science Research Council Grant (A.B.). We are grateful to Klubban Biological Station and Kristineberg Marine Station for field-work facilities and housing. This work complied with all United States and Swedish laws pertaining to the use of *S. typhle* for scientific research.

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Communicated by J.D. Reynolds